IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of	:	ANDERSON, ET AL.
Serial No.	:	904,662
Filed:	:	SEPTEMBER 8, 1992
For	:	GENE THERAPY
Group	:	1804
Examiner	:	JACQUELINE STONE

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OCT 19 1993
GROUP 180

Honorable Commissioner of Patents
Washington, D.C. 20231

SIR:

W. French Anderson declares as follows:

1. He is one of the inventors of the referenced application.

2. He has attached hereto as Exhibit 1 a list of human gene therapy protocols, and to the best of his information and belief, the protocols listed as 1-38 have been approved by the Recombinant DNA Advisory Committee (RAC), a committee of the National Institutes of Health. All of such protocols (except for protocols No. 1-4, which are the protocols of the present application) were approved by the RAC after the above-named inventors had demonstrated the feasibility of human gene therapy through the ADA protocol of the present application.

3. To the best of his information and belief, RAC approval of the human gene therapy protocols 5-38 was obtained in a time period, which on the average was six months or less whereas it took over 3 years to obtain RAC approval for Anderson's initial gene therapy protocol (protocol 1, the ADA protocol of this application): April 24, 1987 (the date of the original pre-protocol submission to the Human Gene Therapy Subcommittee of the RAC)

to July 31, 1990. Now, most protocols are approved after an approximately eight week RAC review.

4. The approved protocols 5-38 are directed to human gene therapy with a variety f DNA sequences, empl ying a variety of delivery vehicles, and are directed to both ex vivo and in situ (in vivo) transduction of human cells. Thus, for example, such protocols include the following:

1. TNF, which is a secreted cytokine
2. IL-2, a secreted lymphokine;
3. LDL receptor, a membrane protein;
4. TK, an activatable viral gene;
5. HLA-B7, a cell surface antigen;
6. HIV-gp120, a surface antigen;
7. IL-4, a cytokine;
8. antisense-RAS, an antisense molecule to an oncogene;
9. p53, a tumor suppressor gene;
10. CF, an integral membrane transport protein;
11. GM-CSF, a hematopoietic colony-stimulating factor;
12. gamma interferon, a cytokine;
13. MDR, a membrane transport protein;
14. glucocerebrosidase, an intracellular enzyme;
15. mutated HIV, a viral protein;
16. Rev, a viral transcription factor;
17. anti-IGF-1, an antisense molecule to a cell growth factor; and
18. ribozyme, an RNA-cleaving RNA molecule.

In addition, the RAC-approved protocols encompass a wide variety of delivery means, such as retroviral vectors, adenovirus vectors, liposomes for delivery of plasmid DNA, and viral-producer cells.

In addition, such RAC-approved protocols encompass both ex-vivo transduction and in situ (in vivo) transduction of cells. Thus, for example, the TK protocol involves the use of a producer cell, which transduces cells in vivo.

The 5 CF protocols involve intratracheal r intranasal infusi n f an adenovirus vector for transduction of cells in vivo.

In addition, such RAC-approved protocols include a protocol f r direct injection into a cancer mass for transduction of cells in vivo.

5. To the best of his information and belief, the RAC does not approve a human gene therapy protocol unless there is a reasonable expectation of efficacy. In his opinion, the rapid approval of the human gene therapy protocols 5-38, in large part, resulted from the fact that the inventors of the present application had demonstrated the feasibility of human gene therapy through protocol 1, the ADA protocol of the present application. In particular, the demonstration of the feasibility of human gene therapy through the ADA protocol indicated that concerns, such as those raised by the Examiner on page 3 of the Office Action in the present application, with respect to the inappropriateness of human gene therapy, had been obviated, whereby it was now possible for those skilled in the art to design and obtain RAC approval for a wide variety of human gene therapy protocols.

6. Prior to the inventors demonstration of the feasibility of human gene therapy, there were no approved human gene therapy protocols. After such demonstration of the feasibility of human gene therapy, in a period of less than three years, there exists 37 additional approved human gene therapy protocols in the United States. In his opinion, the design and approval of such human gene therapy protocols was enabled by the inventors demonstration that human gene therapy is feasible.

7. He has attached hereto as Exhibit 2 a summary of the Institutions involved with human gene therapy protocols.

8. He has attached hereto as Exhibit 3 a graph which indicates the cumulative number of patients through the early part of 1993 who have received gene therapy. The first point n the therapy curve is the first patient on the ADA protocol of the present application. The graph of Exhibit 3 is incomplete in that it covers only to May 1, 1993. The graph illustrates the significant increase in human gene therapy patients, and the rapidity of such increase, after the inventors demonstrated the feasibility of human gene therapy.

9. He declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

W. French Anderson

W. FRENCH ANDERSON

Dated: October 15, 1993

EXHIBIT 1

Human Gene Therapy Clinical Protocols

1. ADA Deficiency - NIH
2. TNF/TIL/Melanoma - NIH
3. TNF/Cancer - NIH
4. IL-2/Cancer - NIH
5. Liver LDL Receptor/FH - Univ. of Michigan
6. TK/Ovarian Cancer - Univ. of Rochester
7. HLA-B7/Melanoma:1 - Univ. of Michigan
8. CTL/TK/AIDS - Univ. of Washington
9. IL-2/Neuroblastoma - St. Jude
10. TK Producer Cells/Brain Cancer - NIH

11. IL-2/Melanoma - Sloan-Kettering
12. IL-2/Renal Cell Cancer - Sloan Kettering
13. HIV gp120/AIDS:2 - Viagene
14. IL-4/Cancer - Univ. of Pittsburgh
15. Antisense Ras/p53/Lung Cancer - M.D. Anderson Hospital
16. CF/Lung/Adenovector - NIH
17. CF/Lung/Adenovector - Univ. of Michigan
18. CF/Nasal Epithelium/Adenovector - Univ. of Iowa
19. TK Producer Cells/Brain Cancer - Iowa Methodist
20. GM-CSF/Renal Cell Cancer - Johns Hopkins

21. CF/Lung/Adenovector - Univ. of Cincinnati
22. CF/Lung/Adenovector - Univ. of North Carolina
23. Gamma-Interferon/Melanoma - Duke University
24. MDR/Ovarian Cancer - M.D. Anderson Hospital
25. HLA-B7/Cancer:2 - Univ. of Michigan
26. Glucocerebrosidase/Gaucher - Univ. of Pittsburg
27. Glucocerebrosidase/Gaucher - NIH
28. HIV-IT(V)/AIDS - Univ. of Southern California
29. Rev-/AIDS - Univ. of Michigan
30. TK Producer Cells/Pediatric Brain Cancer - CHLA

31. MDR/Cancer - Columbia University
32. Anti-IGF-1/Cancer - Case Western Reserve
33. Il-2/Cancer - UCLA
34. MDR/Breast Cancer - NIH
35. Il-2/Melanoma - Univ. of Illinois
36. Il-2/Small Cell Lung Cancer - Univ. of Miami
37. TK Producer Cells/Pediatric Brain Tumor - St. Jude
38. Ribozyme/AIDS - UCSD

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39. HIV gp120/AIDS:1 - Viagen

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40. Factor IX/ Hemophilia B - Fudan Univ., Shanghai (China)
41. IL-2/Cancer - University Hospital, Leiden (Netherlands)
42. ADA Deficiency - San Raffaele Sci. Inst., Milan (Italy)
43. ADA Deficiency - TNO, (The Netherlands + France, England)

Date: 10/1/93

EXHIBIIT 2

SUMMARY

Human Gene Marker/Therapy Clinical Protocols

Number of Marker Protocols: 21
Number of Therapy Protocols: 43
Total Number of Protocols: 64

Institutions: 29

NIH	13	(5+8)
St. Jude	7	(5+2)
M.D. Anderson	5	(3+2)
Univ. of Michigan	5	(0+5)
Univ. of Washington	4	(3+1)
Univ. of Pittsburgh	3	(1+2)
Viagen	2	(0+2)
Sloan-Kettering	2	(0+2)
USC	2	(0+2)
UCLA	2	(1+1)

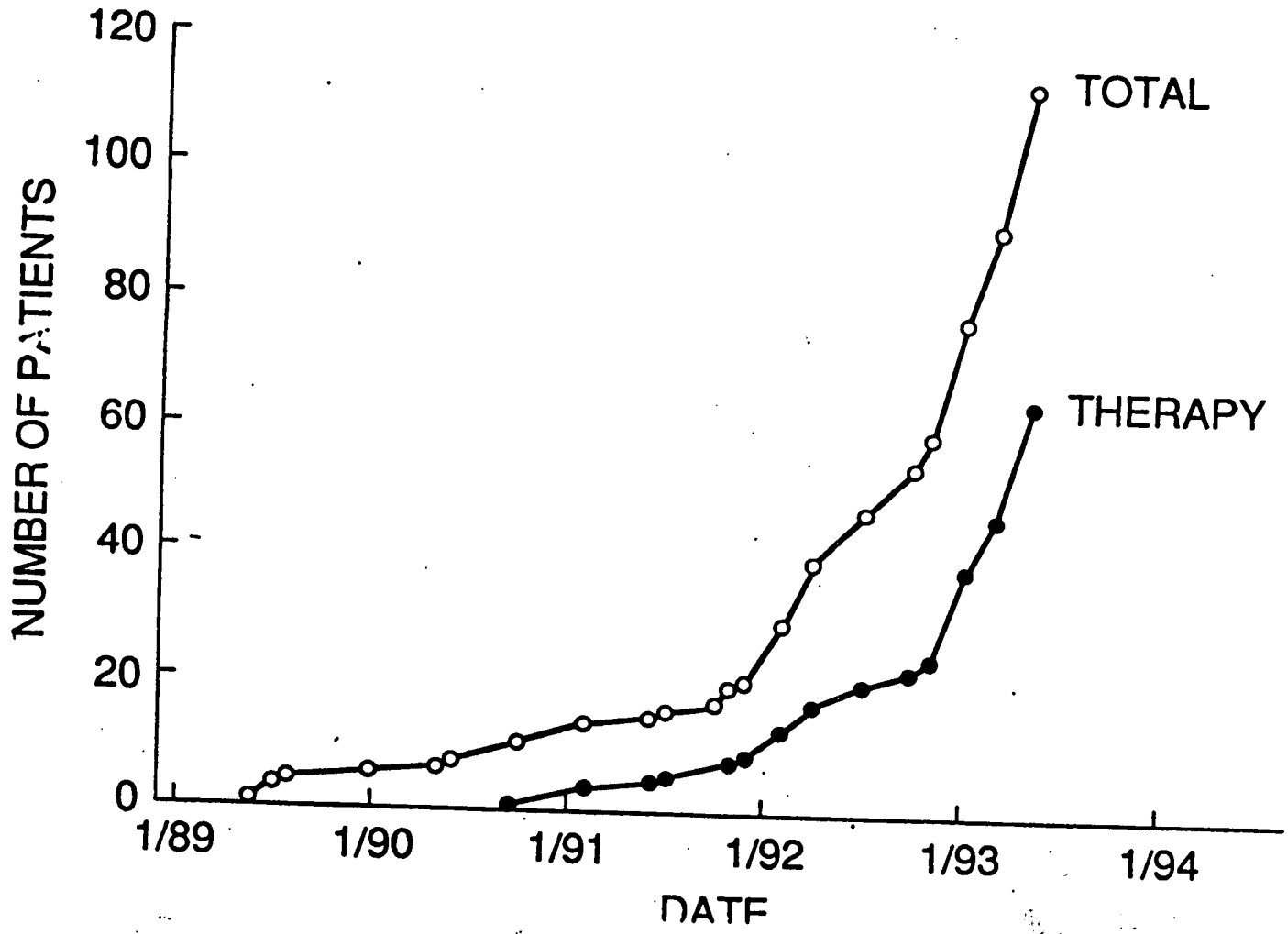
One each:

Marker:	Therapy:
CLB, Lyon	Fudan University, Shanghai
Indiana	University Hospital, Leiden
	SRM, Milan
---	TNO, The Netherlands (Eng, Fr)
Baylor	---
	Univ. of Rochester
	Univ. of Iowa
	Iowa Methodist
	Johns Hopkins
	Univ. of Cincinnati
	Univ. of North Carolina
	Duke University
	Columbia University
	Case Western Reserve
	UC San Diego
	Univ. of Miami
	Univ. of Illinois, Chicago

10/1/93

EXHIBIT 3

GENE MARKER/THERAPY CLINICAL PROTOCOLS: CUMULATIVE NUMBER OF PATIENTS



Proc. Natl. Acad. Sci. USA
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Genetics

Gene therapy for long-term expression of erythropoietin in rats

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Communicated by Eloise R. Giblett, Puget Sound Blood Center and Program, Seattle, WA, May 31, 1995

ABSTRACT **The injection of recombinant erythropoietin (Epo) is now widely used for long-term treatment of anemia associated with chronic renal failure, cancer, and human immunodeficiency virus infections. The ability to deliver this hormone by gene therapy rather than by repeated injections could provide substantial clinical and economic benefits. As a preliminary approach, we investigated in rats the expression and biological effects of transplanting autologous vascular smooth muscle cells transduced with a retroviral vector encoding rat Epo cDNA. Vector-derived Epo secretion caused increases in reticulocytes, with peak levels of 7.8–9.6% around day 10 after implantation. The initial elevation in reticulocytes was followed by clinically significant increases in hematocrit and hemoglobin for up to 11 weeks. Ten control and treated animals showed mean hematocrits of 44.9 ± 0.4% and 58.7 ± 3.1%, respectively ($P < 0.001$), and hemoglobin values of 15.6 ± 0.1 g/dl and 19.8 ± 0.9 g/dl, respectively ($P < 0.001$). There were no significant differences between control and treated animals in the number of white blood cells and platelets. Kidney and to a lesser extent liver are specific organs that synthesize Epo in response to tissue oxygenation. In the treated animals, endogenous Epo mRNA was largely down regulated in kidney and absent from liver. These results indicate that vascular smooth muscle cells can be genetically modified to provide treatment of anemias due to Epo deficiency and suggest that this cell type may be targeted in the treatment of other diseases requiring systemic therapeutic protein delivery.**

Erythropoietin (Epo) is a 30-kDa glycoprotein hormone that serves as the primary regulator of red cell production in mammals (1, 2). The therapeutic potential for Epo in the treatment of anemia associated with renal failure was demonstrated initially by its administration to anemic uremic rats and sheep (3, 4). The availability of recombinant human Epo provided a major advance in the treatment of anemia in renal failure patients receiving dialysis (5). The attendant dangers of transfusion therapy were eliminated and the quality of life of these patients was significantly improved. This treatment, given two or three times weekly, raises hematocrit and hemoglobin levels and improves cardiovascular status (2, 6).

Adenoviral vectors have been used to achieve *in vivo* Epo gene transfer (7, 8). Studies of Epo gene transfer using transplantation of transduced cells have included myoblasts in mice (9, 10) and smooth muscle cells in rats (11). Vascular smooth muscle cells provide an attractive target tissue for gene therapy and have been studied by several investigators (12–17). These cells are easily obtained and cultured and can be efficiently infected with replication-defective retroviral vectors and returned to the donor by seeding in natural or synthetic blood vessels (11–14, 18). Since quiescent smooth muscle cells have a low turnover rate, their implants have the potential to survive and provide therapeutic gene expression for years (13, 19). Furthermore, the proximity of the transduced cells to the circulation may increase their therapeutic usefulness, especially for the systemic secretion of hormones. In previous work, we have shown that rat smooth muscle cells will express transduced marker genes for at least 1 year with no evidence of vector inactivation (13). This suggests the use of genetically modified vascular smooth muscle cells for systemic delivery of regulatory proteins. To pursue this potential clinical application of gene therapy, we investigated in rats the secretion of Epo by genetically modified vascular smooth muscle cells.

MATERIALS AND METHODS

Retroviral Vectors. The retroviral vector LrEPSN was made by inserting an *Eco*RI/*Bam*HI fragment of the rat Epo cDNA into viral plasmid LXSN (20). A plasmid containing the rat Epo gene was kindly provided by Boissel and Bunn (21). The control retroviral vector LASN encoded human adenosine deaminase (ADA) (22).

Cell Culture, Transduction, and Transplantation. Ecotropic PE501 and amphotropic PA317 retrovirus packaging cell lines (20, 23), NIH 3T3 thymidine kinase-negative cells (23), and primary cultures of rat smooth muscle cells were grown in Dulbecco–Vogt-modified Eagle's medium with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum in humidified 5% CO_2/95% air at 37°C.

Rat smooth muscle cell cultures were prepared by enzymatic digestion of the aorta from male Fisher 344 rats. These cells were characterized by positive staining for muscle cell-specific actins with HHF35 antibody (14) while staining negative for von Willebrand factor (14), an endothelial cell-specific marker. Early passage smooth muscle cells were exposed to 16-hr virus harvests from PA317-LrEPSN and PA317-LASN amphotropic virus-producing cell lines for a period of 24 hr in the presence of Polybrene (4 μg/ml). Vascular smooth muscle cells infected with LrEPSN and selected in G-418 antibiotic (1 mg/ml) secreted 6.7 milliunits per 24 hr per 10^5 cells of Epo. For these assays we used an ELISA procedure constructed to measure human Epo (R&D Systems), which probably underestimated the rat hormone. Biological activity of vector-encoded Epo was confirmed by proliferation of a murine erythroleukemia cell line (HCD-57) sensitive to recombinant human Epo (24). Transduced Epo-secreting smooth muscle cells showed the same growth characteristics as control cells both *in vitro* and *in vivo*, indicating the absence of any Epo-mediated autocrine effect (data not shown).

For cell seeding, rats were anesthetized, and the left carotid artery was temporarily isolated with ligatures and denuded of endothelium by passage of a balloon catheter introduced through an arteriotomy in the external branch (12, 13). Transduced vascular smooth muscle cells (10^6 cells in 50 μl of culture medium) were infused over 15 min into the isolated carotid segment by means of a cannula in the external carotid segment

Abbreviations: Epo, erythropoietin; ADA, adenosine deaminase; WBC, white blood cell(s).

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USSN 08/217,324 Exhibit 2

after a brief irrigation with culture medium. The external carotid segment was ligated after removal of the catheter, blood flow was restored, and the wound was closed (12, 13). Anticoagulated blood samples (100 μl) were obtained from the tail vein, and reticulocyte count was determined by vital staining with brilliant cresyl blue and counting 1000 cells by standard techniques. Hematocrit, hemoglobin, platelet, and white blood cell (WBC) number were measured with a Coulter Counter.

Epo mRNA Analysis. Total RNA was isolated from rat liver and kidney after homogenization in the presence of RNazol. Using Moloney murine leukemia virus reverse transcriptase, 1 μg of total RNA was reverse-transcribed in the presence of random hexamer primers. PCR (35 cycles) was performed with rat Epo-specific primers (5′-AGG CGC GGA GAT GGG GGT GC-3′ and 5′-CCC CGG AGG AAG TTG GAG TAG-3′) to give a 540-bp amplified segment. An aliquot of the amplified reaction mixture was electrophoresed in a 2% agarose gel and, after Southern transfer, the membrane was hybridized with a ^{32}P-labeled 500-bp Epo cDNA probe. As a control for RNA extraction, reverse transcription, and amplification, actin-specific primers (5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′) were used to amplify a 500-bp fragment from reverse-transcribed RNA.

RESULTS

In animals seeded with LrEPSN cells, the reticulocyte counts increased, with peak levels of 7.8–9.6% at about day 10 (Fig. 1*A*). In comparison, the reticulocyte counts of control animals, which were seeded with cells transduced with a vector encoding human ADA (LASN) or were subjected to balloon injury alone, did not show changes in reticulocyte counts (Fig. 1*A*). The mean reticulocyte count from 9 control rats was 2.9 ± 0.6%, in agreement with published values, which range from 1.5% to 3.5% (25). Hematocrit and hemoglobin levels in the treated rats gradually increased, reaching peak values at ≈3 weeks that were sustained for up to 79 days (Fig. 1 *B* and *C*). Comparison of blood cell measurements of 10 control and 10 treated rats from day 20 (when peak values were established) showed maintenance of highly significant elevations in the treated group ($P < 0.001$). The hematocrit had a mean increase of 13.8%, and hemoglobin had a mean increase of 4.2 g/dl (Table 1). WBC and platelet values were not different between the control and treated animals ($P = 0.1$). The control hematological levels were in agreement with normal rat blood values (25).

To determine whether vector-encoded Epo expression resulted in down regulation of endogenous Epo production (26), test rats were sacrificed when elevated hematocrit and hemoglobin levels were established. At a similar time point, control rats that received LASN-transduced cells also were sacrificed. RNA harvested from liver and kidney was subjected to reverse transcription PCR using rat Epo-specific probes (21). As shown in Fig. 2, endogenous Epo mRNA in the kidney of a rat seeded with LrEPSN-transduced cells was greatly reduced in comparison to a control kidney. Also, the hybridization signal was below the level found in control liver, the secondary tissue for Epo production. Epo mRNA was undetectable in treated rat liver. These results were confirmed in three other treated rats (data not shown). Southern band intensities from actin mRNA amplification of test and control tissues were similar, indicating equivalence in RNA isolation and amplification (data not shown). The large difference in hybridization signal between kidney and liver in control rat reflects the major and minor contribution, respectively, of these tissues to Epo biosynthesis.

To estimate the number of seeded transduced cells, we determined the secretion of Epo from carotid arteries removed from rats showing elevated blood counts. Two rats that received LrEPSN-transduced cells and two LASN cell seeded controls were sacrificed 2 months after transplantation. Their

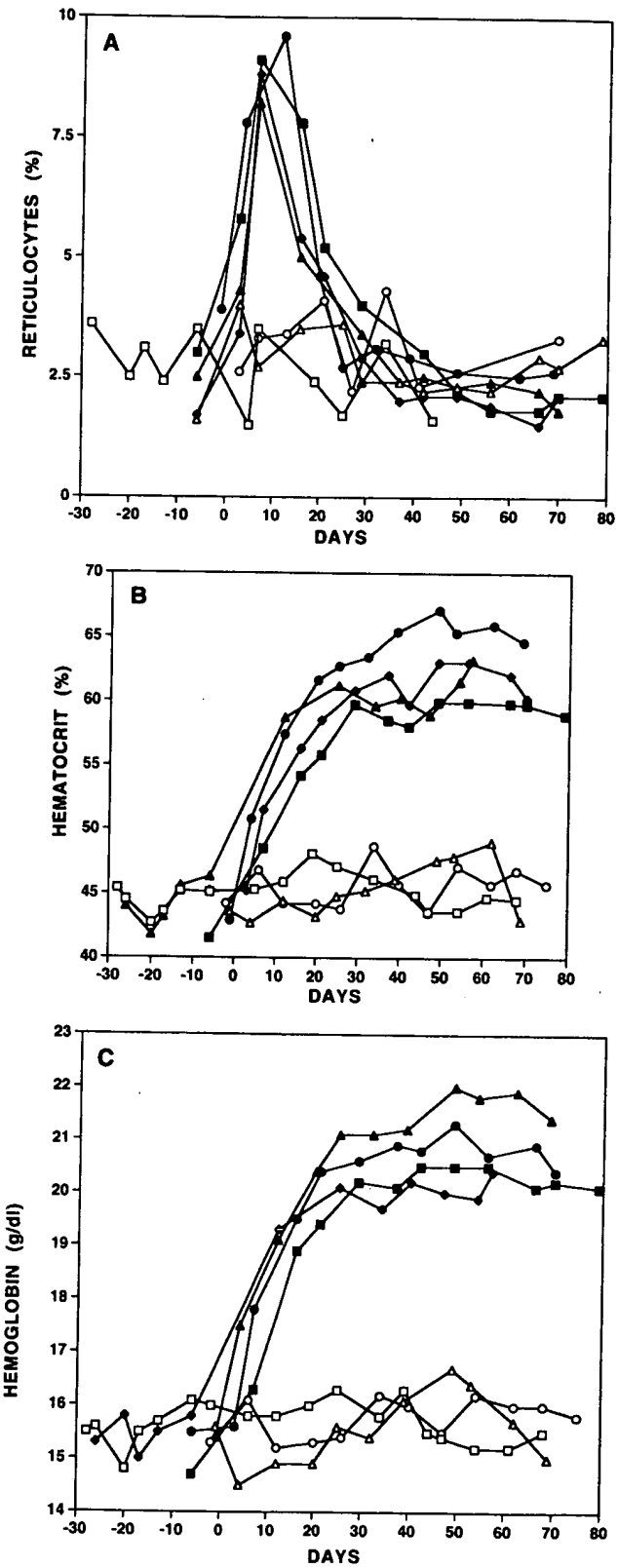

FIG. 1. Effect of seeding transduced vascular smooth muscle cells on reticulocytes (*A*), hematocrit (*B*), and hemoglobin (*C*). Solid symbols, animals seeded with LrEPSN-transduced cells; open symbols, control rats. □, Control rat balloon injured without seeding; the other two control animals received LASN-transduced cells.

Table 1. Control and treated rat blood values

	Hb, g/dl	Hct, %	WBC per μl, ($\times 10^{-3}$)	Plt per μl, ($\times 10^{-3}$)
Control	15.6 ± 0.1*	44.9 ± 0.4*	8.33 ± 0.76†	708 ± 36†
LrEPSN	19.8 ± 0.9*	58.7 ± 3.1*	7.83 ± 0.61†	654 ± 107†

Hct, hematocrit; Plt, platelets; $n = 10$.
*, $P < 0.001$.
†, $P > 0.1$.

carotid arteries were cultured and Epo secretion was measured by an ELISA constructed for human Epo measurement, which probably underestimates rat hormone. A mean Epo secretion of 8.3 milliunits per 24 hr was measured from the LrEPSN carotid arteries; Epo was not detectable from the controls, consistent with kidney and liver as the only source of Epo biosynthesis (26). Thus, based on the preimplantation level of 6.7 milliunits of Epo secreted from 10^5 cultured transduced cells, we estimated that the seeded carotid arteries contained $\approx 1.2 \times 10^5$ Epo-secreting cells, representing 10% of the cell number incubated in the seeding procedure. These data indicate a relatively efficient seeding procedure that results in a cell mass capable of providing sustained gene delivery at therapeutically significant levels.

DISCUSSION

Kidney and to a lesser extent liver are the specific organs that synthesize Epo in response to tissue oxygenation (1, 26, 27). In treated rats, the level of Epo expression we achieved resulted in significant down regulation of endogenous Epo biosynthesis in kidney and total suppression in liver, indicating that the red blood cell production we induced was mediated by vector-encoded Epo secretion.

In these experiments, the viral long terminal repeat promoter in LrEPSN virus expressed unregulated production of Epo. However, control elements from the human Epo gene have been defined (28–31) and if incorporated in a retroviral vector may allow delivery of a regulated supply of Epo. Hypoxia regulates Epo synthesis primarily through the rates of gene transcription in the kidney (1). The hypoxia-responsive cis elements of the Epó gene are localized to the 3′ region (29–35) and have been shown to interact with the Epo promoter (31). Of potential interest is the fact that the oxygen-sensing system initially identified in Epo-producing cells has been found in a wide variety of cell types (33–35). However, these up regulatory elements may not be useful with our vector construct, where Epo expression was driven by the strong viral long terminal repeat promoter. We propose, rather, that genetic elements to control potential overproduction of Epo and abnormally high hematocrits would be more appropriate (i.e., negative-regulatory elements), and these are currently not defined in a size suitable for insertion into a retroviral vector (36).

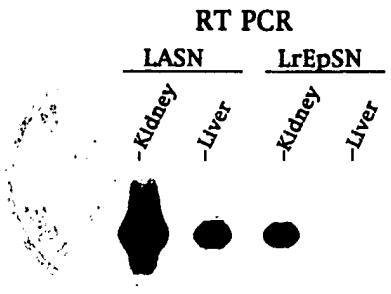

FIG. 2. Epo mRNA analysis. Total RNA was isolated from kidney and liver of rats receiving Epo-expressing LrEPSN-transduced cells and control rats receiving LASN-transduced cells expressing human ADA. Reverse transcription (RT) PCR was performed with rat Epo-specific primers to give a 540-bp amplified segment that was subjected to electrophoresis and hybridized with a ^{32}P-labeled Epo probe.

We observed no significant differences in WBC and platelet numbers between treated and control rats, indicating that constitutive vector-encoded Epo secretion enhanced red cell production without other hematological effects. A previous study reported decreased platelet counts in mice receiving large, chronic injections of Epo (37). However, the majority of human studies report no significant changes in leukocyte or megakaryocyte production from the long-term administration of recombinant Epo (5, 24, 38).

The constitutive level of Epo we achieved in this study would provide useful therapy for patients with renal failure. Although arterial seeding is not feasible in human subjects, we have recently shown in baboons that prosthetic vascular grafts can be used as a device to implant transduced cells (14). From the data produced in this rat model and our studies in dogs and baboons (14, 18), we estimate that 10^8 transduced vascular smooth muscle cells can provide a therapeutic dose of Epo to an 80-kg patient, and this cell number could be transplanted in a 10 cm × 4 mm prosthetic graft. Such grafts are frequently used to provide dialysis access for patients with renal failure (2) and could be readily seeded with genetically modified autologous smooth muscle cells to secrete Epo. The estimated annual cost of recombinant Epo for the 85,000 patients in the United States with anemia of end-stage renal disease is in excess of $500 million (39). The ability to treat these patients, and others with Epo-responsive anemias, by gene therapy would provide major clinical and economic benefits.

These studies have demonstrated that gene therapy targeted at vascular smooth muscle cells may provide a useful approach to the treatment of anemias due to Epo deficiency and other diseases, such as the hemophilias, that are responsive to the administration of regulatory proteins.

We thank Drs. J.-P. R. Boissel and H. F. Bunn for kindly supplying the rat Epo cDNA and Dr. J. L. Spivak for cell line HCD-57. This work was supported by National Institutes of Health Grants DK 43727 and DK 38531 to W.R.A.O. and HL 42270 and HL 18645 to A.W.C.

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Gene Transfer in Baboons Using Prosthetic Vascular Grafts Seeded with Retrovirally Transduced Smooth Muscle Cells: A Model for Local and Systemic Gene Therapy

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ABSTRACT

Prosthetic vascular grafts containing retrovirally transduced autologous vascular smooth muscle cells were studied as a model for introduction of human genes into baboons. Retroviral vectors encoding β-galactosidase (β-Gal) (LNPoZ) or human purine nucleoside phosphorylase (LPNSN-2), a control gene, were used for *ex vivo* transduction of autologous baboon smooth muscle cells obtained from vein biopsies. Transduced cells were placed into a collagen solution and seeded into the interstices of polytetrafluoroethylene vascular grafts. Endothelial cells were then seeded onto the luminal surface of the grafts to reduce thrombus formation. One LNPoZ-seeded graft and one LPNSN-2-seeded control graft were implanted bilaterally into the aorto-iliac circulation of each of 4 animals. All grafts remained patent until they were removed after 3–5 weeks and examined histochemically for vector-expressing cells. All histological cross-sections from the β-Gal vector seeded grafts contained cells staining blue with the X-Gal chromogen. For the four grafts, the mean fraction of LNPoZ expressing cells was 10%, with a range of 2–20%, while no sections from the control grafts contained stainable cells. Smooth muscle cells expressing the reporter gene were localized within the graft wall but not in the newly forming intima or outer capsule of fibrous tissue. Implantation of transduced cells within this type of vascular graft may provide a useful approach for long-term local and systemic gene therapy.

OVERVIEW SUMMARY

Prosthetic (PTFE) vascular grafts seeded with transduced autologous vascular smooth muscle cells were introduced into baboons. Cell seeding was accomplished by the intercalation of smooth muscle cells into the interstices of the PTFE graft matrix. Vascular endothelial cells were seeded onto the luminal surface of the grafts to provide an antithrombogenic surface. Grafts were implanted into the aorto-iliac circulation of 4 animals and at up to 5 weeks β-Gal-expressing cells were observed in all grafts treated with LNPoZ-transduced cells; all grafts remained patent. This strategy for the implantation of genetically modified vascular smooth muscle cells may provide a useful approach to long-term local and systemic gene therapy.

INTRODUCTION

SMOOTH MUSCLE CELLS (SMC) are the predominant cell type within the vasculature and exist as a multilayered mass of long-lived cells in proximity to circulating blood. SMC play a critical role in arterial growth and development as well as in vascular diseases (Schwartz *et al.*, 1990; Ross 1993). These characteristics have led us and others to explore the use of SMC as vehicles for gene therapy (Nabel *et al.*, 1990; Plautz *et al.*, *1991; Lynch et al.*, 1992; Clowes *et al.*, 1994). SMC provide an ideal target tissue for retroviral-mediated gene transfer because they are readily obtained, cultured, infected, selected, and transplanted (Clowes *et al.*, 1994). Retroviral vectors ffer a safe and effective method of introducing and expressing recombinant genes in a variety of cell types (Miller, 1992; Mor-

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gan and Anderson, 1993; Salmons and Gunzburg, 1993). Recently, long-term vector-encoded gene expression from retrovirally transduced SMC has been achieved in rodents (Lynch *et al.*, 1992; Clowes *et al.*, 1994). Autologous rat SMC transduced with human genes were seeded into rat carotid arteries denuded of endothelium, and vector expression was documented beyond 1 year (Clowes *et al.*, 1994). However, rodent models of gene therapy are not necessarily transferable to large animals (Miller, 1990), and, furthermore, it is unlikely that seeding of cultured cells into denuded arteries will be feasible in patients. Therefore, other strategies to transplant genetically modified SMC in numbers adequate for local or systemic effects are needed.

We have previously characterized a model of prosthetic vascular graft healing in non-human primates (Clowes *et al.*, 1985, 1986a; Clowes and Reidy, 1987). In this model, SMC and endothelial cells that populate the porous polytetrafluoroethylene (PTFE) graft wall and neointima are derived from two sources; the cut arterial edge at each anastomosis and the rich microvascular network in the granulation tissue surrounding the graft (Clowes *et al.*, 1986b). The ingrowth of these cells results in the formation of a neointima composed of SMC and extracellular matrix underneath an endothelial monolayer. We hypothesized that preseeding PTFE grafts with retrovirally transduced SMC might provide clinically relevant numbers of cells capable of expressing recombinant genes long term. To reduce the risk of thrombus formation, the luminal surface of such grafts can be seeded with endothelial cells. Also, this "cassette" of porous PTFE graft and transduced SMC could be removed and replaced if necessary and, by adjusting graft length and cell density, the amount of gene product might be regulated. This report outlines our initial success with this approach in primates, suggesting that it may be applied to provide local and systemic gene therapy in patients.

MATERIALS AND METHODS

Retroviral vectors

Vectors encoding the selectable neomycin phosphotransferase (neo) either with the reporter gene *Escherichia coli* β-galactosidase (β-Gal) or a control gene, human purine nucleoside phosphorylase (PNP), were constructed as previously described (Osborne and Miller 1988; Miller and Rosman 1989; Adam *et al.*, 1991). Nomenclature of these vectors (LNPoZ and LPNSN-2, respectively) is based on the order of genetic elements: L, LTR promoter; N, neo; Po, poliovirus IRES 5' sequences; Z, *lacZ* (β-Gal) gene; PN, PNP; S, simian virus 40 promoter. Amphotropic retroviral vectors were produced from PA317 packaging cells (Miller and Buttimore, 1986). LNPoZ producer cells were titered at 5×10^5 colony-forming units (cfu)/ml (Adam *et al.*, 1991) and the LPNSN-2 producer line at 2×10^6 cfu/ml (Osborne and Miller, 1988).

Primary autologous SMC and endothelial cell culture

Endothelial cells and SMC were both obtained following a single vein biopsy from each baboon. Under general anesthesia, an 8-cm length of lesser saphenous vein was excised, side branches were ligated, and the ends were cannulated to facilitate flushing. After rinsing the lumen with 5 ml of Hank's balanced salt solution [(HBSS), GIBCO, New York, NY], 3 ml of a Dispase solution was introduced (2.4 U/ml in HBSS, Boehringer Mannheim Inc., Indianapolis, IN) to remove the endothelial cells selectively. The vein was slightly distended with the enzyme solution by occluding venous outflow and then incubated at 37°C for 20 min. Released endothelial cells were gently flushed from the vein lumen with 10 ml of HBSS, spun, plated onto gelatin-coated dishes, and cultured in RPMI-1640, 20% FBS (vol/vol) containing 90 μg/ml of heparin sodium and 100 μg/ml of endothelial cell growth supplement (Collaborative Research, Bedford, MA) in a humidified incubator with 5% CO_2/95% air at 37°C.

After endothelial cell harvest, SMC were removed from the same vein either by enzyme digestion or outgrowth from cultured pieces of vein. For digests, de-endothelialized vein segments were stripped of adventitia and treated with collagenase-P (1 mg/ml; Boehringer-Mannheim), elastase (2 mg/ml; Boehringer-Mannheim), soybean trypsin inhibitor (400 μg/ml; Worthington), and bovine serum albumin (1 mg/ml; Sigma). Explants were prepared by plating 2-mm^2 pieces of de-endothelialized vein in Dulbecco modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS) (vol/vol), incubated at 37°C, 5% CO_2. When outgrowth of SMC were obtained, tissue fragments were removed and the cells expanded.

Cultured cells were characterized immunocytochemically with cell-specific primary antibodies localized with fluorescein-conjugated secondary antibodies. SMC stained positive with antibodies against α-actin (1:100 dilution, Boehringer Mannheim, Indianapolis, IN) and vimentin (1:25, Dako, Carpenteria, CA) but negative for the endothelial cell marker, factor VIII-related antigen (1:100, Dako). Endothelial cells stained in a reciprocal fashion.

SMC transduction

Early passage (P1 or P2) SMC were exposed to viral harvests from the murine amphotropic virus-producing cell lines, PA317/LNPoZ and PA317/LPNSN-2, for a period of 24 hr in the presence of Polybrene (4 μg/ml, Sigma). Infected cells were selected in G418 (1 mg/ml; GIBCO) for 10–14 days and then expanded in the absence of G418 for seeding. When at least 5×10^7 of both LNPoZ and LPNSN-transduced SMC were available from an individual baboon the cells were prepared for graft seeding.

PTFE graft preparation

Prior to seeding, transduced SMC were harvested, counted, and placed into 5 ml DMEM containing heat-inactivated (56°C f r 30 min) autologous serum (25% vol/vol) and 0.75 mg/ml type-I collagen (Vitrogen 100, Celltrix, CA). The SMC–collagen suspension was kept cool until used. Using sterile technique, reinforced porous PTFE grafts (W.L. Gore and Associates, Inc., Flagstaff, AZ) of 4 mm internal diameter and 10 cm in length were immersed in 95% ethanol and then flushed three times with 5 ml of phosphate buffered saline (PBS). One end of the wetted graft was occluded and the SMC–collagen suspen-

sion was introduced from the other end with a 5-ml syringe and filtered gently through the graft wall. The filtrate was collected and refiltered a total of four to six times. Grafts were then unclamped, drained, and warmed at 37°C for up to 45 min until the collagen polymerized. Grafts were then covered with media and incubated overnight. The following morning, endothelial cell cultures were harvested and suspended in 2 ml of media with 10% heat-inactivated autologous serum. The endothelial cell suspension was introduced into the lumen of the SMC-seeded grafts and the ends were clamped. Grafts were rolled 180° at 10-min intervals over 40 min to distribute the endothelial cells evenly and provide a nonthrombogenic luminal surface. Treated grafts were then delivered to the surgical suite for immediate implantation.

Animal model

Seeded grafts (one LNPoZ and one LPNSN-2) were placed into the aorto–iliac circulation of 4 young male baboons (*Papio cynocephalus*) weighing ~10 kg. Anesthesia was induced with intramuscular ketamine hydrochloride (10 mg/kg) and maintained with inhaled halothane. Antibiotics were administered intramuscularly (cefazolin sodium, 25 mg/kg, Bristol-Myers Squibb, Princeton, NJ) and heparin sodium was administered intravenously (200 U/kg, Elkins-Sinn, Inc., Cherry Hill, NJ). Grafts were sutured end-to-side to the infrarenal aorta and to the common iliac artery bilaterally using 6-0 polypropylene suture (Davis and Geck, Danbury, CN). The bypassed native circulation was ligated, diverting all flow through the two grafts. The retroperitoneum and abdomen were closed, wounds infiltrated with bupivacaine hydrochloride (Marcaine 0.25%, Winthrop Pharm., New York, NY) and the animals returned to single-animal cages until they were fully recovered from surgery. Perioperative analgesics included intramuscular ketorolac tromethamine (Toradol, 30 mg load then 15 mg/8 hr, Syntex, Palo Alto, CA) and oral acetominophen (Children's Tylenol, 80 mg/6 hr, McNeil, Fort Washington, PA). Animals received 1.25 grains of aspirin 2 days before surgery and twice weekly thereafter. Animals were sedated weekly with ketamine (10 mg/kg i.m.) for duplex ultrasound interrogation of graft flow and for physical examination. After 3–5 weeks animals were again anesthetized and grafts removed for analysis.

All animal care and procedures were performed at the University of Washington Regional Primate Research Center in accordance with state and federal laws. Animal protocols were approved by the University of Washington Animal Care Committee and conformed to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and by the National Institutes of Health in publication No. 86-23, Guide for the Care and Use of Laboratory Animals.

Histology

Grafts were removed for analysis under general anesthesia at 3 weeks (2 animals), 4 weeks (1 animal), and 5 weeks (1 animal). All grafts were patent, providing one LNPoZ graft and one LPNSN-2 graft from each animal. Heparin was administered and the grafts were excised and immediately rinsed with sterile saline and cut into 0.5-cm rings that were processed alternately into 10% buffered formalin, methyl Carnoy's fixative, and OCT embedding media for frozen sections. For localization of β-Gal-expressing SMC, frozen sections were cut (0.5 μm thickness) from both β-Gal (LNPoZ) and control (LPNSN-2) grafts and mounted onto glass slides. Sections were cut from analogous regions of the two grafts from an individual animal and were processed simultaneously. Slides were immersed in 0.5% glutaraldehyde for 10 min and washed three times in PBS; sections were then covered with 100 μl of the X-Gal chromogen (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 Prime-3, Prime, Inc., Boulder, CO). Slides were then incubated 6 hr at 37°C in a moist chamber, rinsed in PBS, dehydrated in graded alcohols, cover slipped, and examined by standard light microscopy for the presence of blue cytoplasmic staining to indicate β-Gal expression.

To estimate the fraction of LNPoZ-transduced cells in seeded grafts, rings cut from frozen sections were stained with X-Gal, counter stained with hematoxalin, and examined by standard light microscopy. Total cell numbers (hematoxalin-stained nuclei) and vector expressing cells (X-Gal stained cytoplasm) were counted in nonoverlapping high-power fields (630×) viewed radially from the luminal to abluminal graft surface at 16 locations evenly spaced around each graft-cross section. The mean % of vector-positive cells for each graft was obtained by averaging values from four separate cross sections.

To characterize graft cellular composition, paraffin-embedded, methyl Carnoy's fixed sections from each graft were deparaffinized in xylene, rehydrated in graded alcohols, and immunostained with antibodies specific for SMC α-actin (1:500, Boehringer Mannheim), endothelial cell Factor-VIII related antigen (1:500, DAKO), and macrophage CD-68 (1:1000, DAKO). Primary antibodies were localized with appropriate biotinylated secondary antibodies and tertiary avidin-biotin-peroxidase staining (Vector Laboratories Inc., Burlingame, CA). Control slides were included using appropriate nonimmune immunoglobulin G (IgG) as the primary antibody. Sections were counterstained with methyl green or hematoxylin and examined using standard light microscopy. Adjacent sections were stained with hematoxylin and eosin for standard histology.

RESULTS

Cell culture, transduction, selection, and expansion

Pure cultures of smooth muscle cells (Fig. 1A) and endothelial cells (Fig. 1B) were obtained from each animal as documented by cell-specific immunofluorescence. Vector expression was monitored from LNPoZ-transduced cells by enzyme specific staining, which revealed intensely blue-colored cells (Fig. 1C). LPNSN-2 transduced cells did not stain with the X-Gal chromogen (data not shown). LPNSN-2 expression was assayed in cultured SMC by histochemical staining after enzyme separation by starch gel electrophoresis, which clearly distinguished human from endogenous baboon PNP (data not shown). Extracts of LNPoZ- and LPNSN-2-transduced SMC had PNP activities of 1.1 μmol/hr mg protein and 9.0 μmol/hr per mg protein, respectively. This represents an eight-fold increase in PNP activity in PNP-vector infected SMC and shows a strong LTR promoter activity in this cell type.

Within 4–5 weeks of obtaining vein bi psies, 5×10^7 transduced SMC were available for graft seeding. Endothelial cells (nontransduced) were available more quickly and were stored under liquid nitrogen until needed for seeding. Trypan blue exclusion assays showed greater than 95% viability of both cell types after completion of the seeding process. Microscopic analysis of seeded graft cross sections immediately prior to implantation showed SMC distributed throughout the graft wall (Fig. 1D).

Histology: immunocytochemical and histochemical SMC localization

Animals remained healthy and all grafts were patent throughout the period of study. Duplex ultrasound examinations demonstrated no significant flow-limiting graft or anastomotic stenoses. At the time of removal, grafts were well incorporated by gross inspection. The luminal surface appeared to be incompletely endothelialized, with scattered islands of thin adherent thrombus.

Histochemical staining with the X-Gal chromogen demonstrated clusters of vector-expressing cells within the graft wall interstitium of each β-Gal (LNPoZ)-seeded graft (Fig. 1E). The fraction of cells expressing β-Gal, determined by analysis of different cross sections, was 6, 7, 13, and 15% for individual animals, respectively (mean, 10 ± 2% [±SEM], range 2–20%). In all grafts, β-Gal-expressing cells were seen in the middle and outer regions of the graft wall. None of the control (LPNSN-2-seeded) grafts stained blue (Fig. 1F). The histological appearance of the two grafts was otherwise indistinguishabl . Vector-expressing cells were not found within the forming neointima or the fibrous tissue outside the graft wall. In addition, β-Gal-expressing cells were not seen to localize in microvessels within the graft wall. In the LPNSN-2-seeded grafts, the presence of red blood cells interfered with the assay of vector-encoded human PNP. Baboon red cell PNP was subject to post-translational modification (Turner *et al.*, 1971) and the resulting multiple isozymes co-migrated with the human PNP-baboon SMC PNP in starch gel (data not shown).

Analysis of graft cross sections by standard light microscopy (H & E stain, Fig. 1G) showed a thin, incompletely endothelialized neointima along the length of β-Gal and control grafts. The interstitium of the grafts was filled with microvessels and a cellular infiltrate. Immunocytochemical staining demonstrated many α-actin-positive SMC (Fig. 1H) in the forming neointima as well as in clusters, in a pattern resembling the distribution of β-Gal-positive cells (Fig. 1E), and in microvessels within the graft wall (Fig. 1H). Macrophages (CD68-positive cells) were seen largely within the PTFE graft wall with few seen in the forming neointima (data not shown). This pattern is typical of healing porous PTFE grafts (Clowes *et al.*, 1986b).

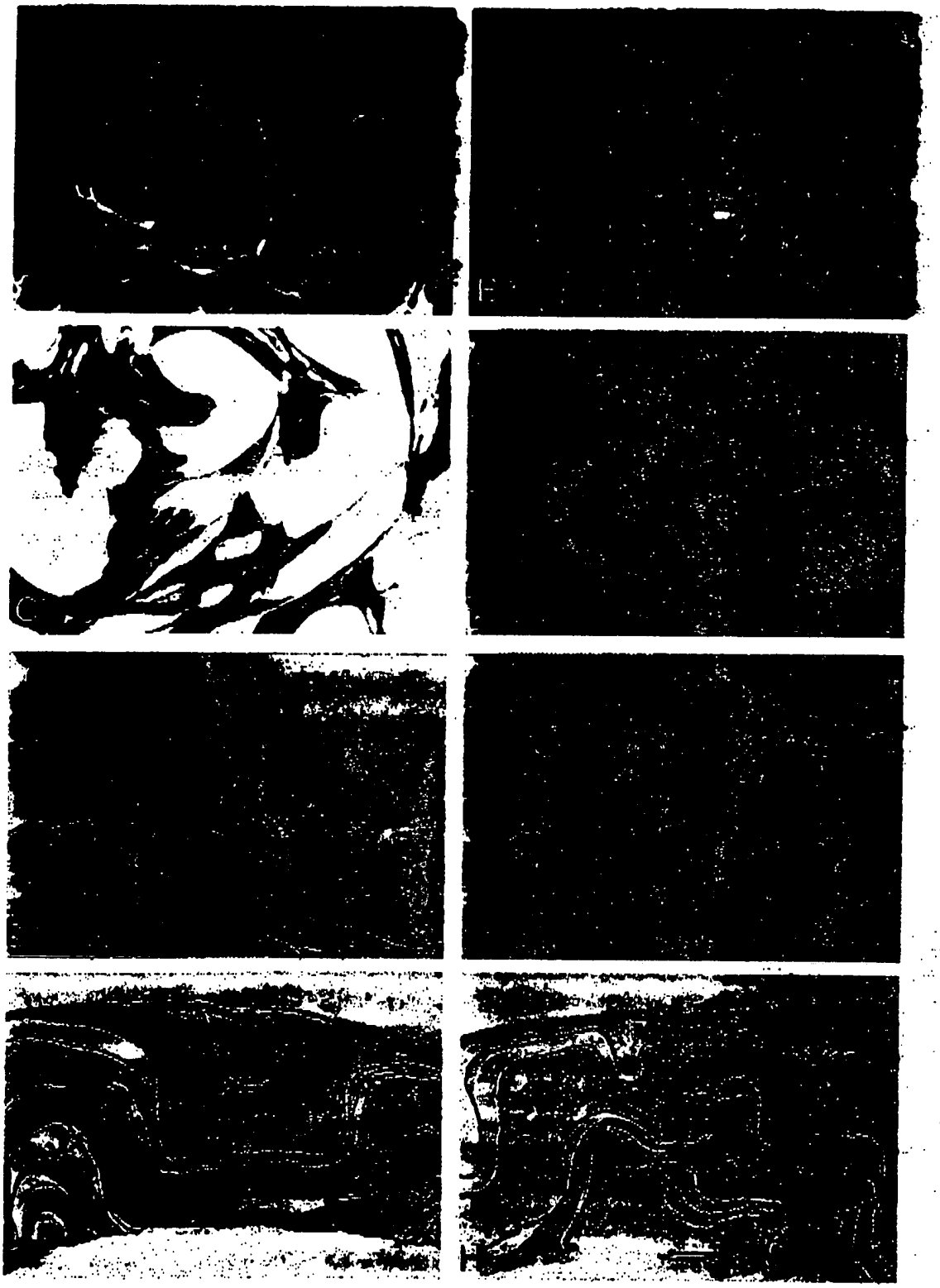
DISCUSSION

Autol gous baboon SMC were transduced and expanded *ex vivo* to clinically relevant numbers (10^7 to 10^9 cells) from small vein biopsies in a relatively brief period of time (4–5 weeks). The genetically modified cells were returned to the d n r baboon circulation within preseeded PTFE vascular grafts coated with endothelial cells. These grafts remained patent and, when removed at up to 5 weeks later, significant numbers of vector-expressing SMC were found in the middle and outer region of the interstices of the PTFE graft wall, with no evidence for migration of transduced cells into the neointima or the area outside of the graft. Histological cross sections taken from the ends of seeded grafts immediately prior to implantation showed that, although cells seed into the largest avenues in the graft, overall a fairly even pattern of cell seeding with slightly more cells near the graft lumen was achieved. These observations suggest that, following implantation, seeded SMC either survived better in the outer graft wall or that they migrated toward the outer graft wall. Grafts removed at 3, 4, and 5 weeks after implantation were similar in appearance, indicating that the events localizing β-Gal-expressing SMC to the mid and outer graft wall occurred early and the graft cell organization then remained stable.

The behavior of transplanted vector-expressing SMC has been characterized in a rat model (Clowes *et al.*, 1994). Transduced SMC seeded into rat carotid arteries decreased in number within the first few days of seeding and the cell number then remained stable to at least 1 year. Seeded SMC coexisted with endogenous SMC that migrated into the forming intima following balloon denudation. Vector-expressing SMC seeded into the rat artery appear to migrate as well as proliferate at early times during the intimal thickening process, and then revert to a quiescient state (Clowes *et al.*, 1994). SMC expressing a reporter gene were found within the layers of arterial media nearest to the lumen and overlying intima in nearly all sections examined. It is of interest that transduced SMC seeded into rat arteries remained in the deeper abluminal region of the intima and the first few underlying layers of media as the intimal thickening developed. This is somewhat analogous to the distribution of transduced baboon SMC in the current study where β-Gal-expressing SMC were found in the mid and outer wall of seeded vascular grafts.

The PNP activities of the cells transduced with LPNSN-2 vector had a high level of human PNP expression, showing that

FIG. 1. SMC cultured from vein biopsies were characterized by α-actin immunofluorescence (A) and endothelial cells by Factor VIII-associated antigen immunofluorescence (B). β-Gal expression was documented in transduced SMC cultures after selection in neomycin phosphotransferase. Virtually all cells stained intensely blue with the X-Gal chromogen (C). A graft seeded with β-Gal-expressing SMC is shown in cross section prior to implantation (D). Many cells staining blue with the X-Gal chromogen are distributed throughout the graft wall. Magnification, A, B, and C, 400×; D, 100×. Graft cross sections from an animal 4 weeks after implantation were stained with the X-Gal chromogen (E and F). Many blue-staining cells were seen in the wall of the graft seeded with the β-Gal-expressing smooth muscle cells (E). These cells are seen in the mid and outer graft wall (top) and not in the inner wall of the graft or the forming neointima (bottom). The control graft from the same animal (seeded with LPNSN-expressing cells) did not stain with X-Gal (F). Both grafts had a similar appearance when stained with hematoxylin & eosin (G) or immunostained for smooth muscle cell α-actin (H). Brown α-actin staining identified SMC throughout the graft wall and clusters of cells resembled those staining positive with X-Gal in the β-Gal-seeded grafts. Magnification, E–H, 100×.



this viral LTR promoter is strongly active in smooth muscle cells. At 5 weeks post-implantation, SMC stained positive for vector-encoded β-Gal expression, indicating that SMC do not inactivate vector sequences *in vivo*, in agreement with studies of rat SMC (Lynch *et al.*, 1992; Clowes *et al.*, 1994).

The vector-expressing SMC population in baboon grafts remained stable in appearance at 3, 4, and 5 weeks following implantation. It is of interest that the seeded SMC remained in the wall of the graft and were not seen to contribute to the forming intima. Perhaps of more significance to gene therapy, seeded SMC were not found in connective tissue outside of the graft wall. This localization makes it feasible to remove the entire population of transduced SMC, if necessary, by simply removing the graft. Techniques for graft implantation are well established for the arterial circulation. Prosthetic grafts are often used to reconstruct diseased arteries when autologous vein is not available and PTFE grafts are very frequently employed in patients with renal failure as a standard conduit for creating arteriovenous fistulae for dialysis access. The close proximity of the genetically modified cells to the circulation suggest this system could be useful for gene therapy involving both secreted and nonsecreted proteins.

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G-CSF EXPRESSION FROM TRANSDUCED VASCULAR SMOOTH MUSCLE CELLS PROVIDES SUSTAINED NEUTROPHIL INCREASES IN RATS.

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ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) regulates granulocyte precursor cell proliferation, neutrophil survival and activation. Cyclic hematopoiesis, a disease which occurs both in man and grey collie dogs is characterized by cyclical variations in blood neutrophils. Although the underlying molecular defect is not known, long-term daily administration of recombinant G-CSF eliminates the severe recurrent neutropenia, indicating that expression of G-CSF by gene therapy would be beneficial. As a prelude to preclinical studies in affected collie dogs we monitored hematopoiesis in rats receiving vascular smooth muscle cells transduced to express G-CSF. Cells transduced with LrGSN, a retrovirus expressing rat G-CSF, were implanted in the carotid artery and control animals received cells transduced with LASN, a retrovirus expressing human adenosine deaminase (ADA). Test animals showed significant increases in neutrophil counts for at least 7 weeks, with mean values of 3670 ± 740 cells/ul in comparison to 1870 ± 460 cells/ul in controls ($P<0.001$). Thus, in rats G-CSF gene transfer targeted at vascular smooth muscle cells initiated sustained production of 1800 neutrophils/ul, a cell number that would provide clinical benefit to patients. Lymphocytes, red cells and platelets were not different between control and test animals ($P>0.05$). These studies indicate that retrovirally transduced vascular smooth muscle cells can provide sustained clinically useful levels of neutrophils in vivo.

OVERVIEW SUMMARY

Cyclic hematopoiesis is a disease which occurs both in man and grey collie dogs and is characterized by cyclical variations in blood neutrophils. Although the underlying molecular defect is unknown, long-term daily administration of recombinant granulocyte colony-stimulating factor (G-CSF) eliminates the severe recurrent neutropenia, indicating that expression of G-CSF by gene therapy would be beneficial. In a preclinical rat model we monitored neutrophil production in rats receiving vascular smooth muscle cells transduced to express G-CSF. Cells transduced with LrGSN, a retrovirus expressing rat G-CSF, were implanted in the carotid artery and control animals received cells transduced with LASN, a retrovirus encoding human adenosine deaminase. Test animals showed a doubling of neutrophil counts for at least 7 weeks, with mean values of 3670 ± 740 cells/ul in comparison to 1870 ± 460 cells/ul in controls ($P<0.001$). This suggests that G-CSF gene transfer targeted at vascular smooth muscle cells mediated production of 1800 cells/ul, a neutrophil count that would provide clinical benefit to patients. These studies show that retrovirally transduced vascular smooth muscle cells can provide sustained clinically relevant levels of neutrophils in vivo.

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that selectively stimulates the proliferation and differentiation of neutrophil precursors and accelerates neutrophil maturation and release from the marrow(Demetri and Griffin 1991 ; Morstyn and Dexter 1993). Recombinant G-CSF has recently been used to treat chronic neutropenias of various causes in order to decrease morbidity and mortality due to infection (Morstyn, et al. 1993). Regardless of cause, treatment of neutropenic individuals with recombinant G-CSF often results in at least a partial normalization of neutrophil number and function. However, due to the symptomatic rather than curative nature of the treatment, these diseases often require lifelong daily injections with this hormone. In this category is cyclic hematopoiesis, a disease which occurs both in man and grey collie dogs, and is characterized by cyclical variations in blood neutrophils, monocytes, lymphocytes, eosinophils, reticulocytes, and platelets due to periodic fluctuations in blood cell production by the bone marrow (Dale et al., 1972 ; Jones and Lange 1983 ; Dale and Hammond 1988). The recurrent severe neutropenia leads to bacterial infections and shortened life expectancy. The disorder can be cured by bone marrow transplantation in grey collie dogs as well as in humans; and in both dogs and humans the disease can be transferred from an affected to a normal (Dale and Graw 1974 ; Weiden et al., 1974 ; Krance et al., 1982). This transplantability strongly supports the concept that this is a disease of defective regulation of hematopoietic stem cells. Although the underlying molecular defect is not known, long-term daily administration of recombinant G-CSF eliminates the severe recurrent neutropenia (Lothrop et al., 1988 ; Hammond et al., 1989 ; Hammond et al., 1990). The constitutive expression of G-CSF by gene therapy would provide clinical and probably economic benefits.

The major colony stimulating factors have been expressed in several cell types following gene transfer in vitro (Lang et al., 1985 ; Laker et al., 1987 ; Wong et al., 1987 ; Browder et al., 1989) , and the retrovirus-mediated transfer and expression of IL-

3 (Wong et al., 1989) , GM-CSF (Johnson et al., 1989) and G-CSF (Chang et al., 1989) in mouse hematopoietic cells in vivo has been described. The retrovirally-expressed IL-3 (Wong, et al. 1989) and GM-CSF (Johnson, et al. 1989) produced a fatal myeloproliferative syndrome in treated mice. Most noteworthy, however, long term expression of G-CSF produced sustained neutrophilia which was not associated with disease (Chang, et al. 1989). Similarly, a mouse mammary tumor constitutively expressing G-CSF produced sustained neutrophilia in mice without myeloproliferative disease (Lee and Lottsfeldt 1984) . The severe recurrent neutropenia in grey collie dogs was not abrogated by in vivo IL-3 or GM-CSF treatment (Hammond, et al. 1990) . IL-3 caused eosinophilia, whereas recombinant human GM-CSF caused neutrophilia and eosinophilia, but with both agents cycling of hematopoiesis persisted (Hammond, et al. 1990) . In contrast, G-CSF prevented the recurrent neutropenia and obliterated periodic fluctuation of monocyte, eosinophil, reticulocyte, and platelet counts (Lothrop, et al. 1988 ; Hammond, et al. 1990) .

We have shown in rats long-term expression of human ADA (Lynch et al., 1992 ; Clowes et al., 1994) , and erythropoietin (Osborne et al., 1995) from transduced smooth muscle cells seeded into carotid arteries. In vascular injury, where the vessel is not completely re-endothelialized, vascular smooth muscle cells form a pseudoendothelium that is in direct contact with the blood (Clowes et al., 1983). Vascular smooth muscle cells are readily obtained, cultured, transduced and implanted, making these cells a generally useful target tissue for gene therapy. We recently cloned rat G-CSF (Han et al., 1996) and as a prelude to the treatment of collie dogs with cyclic hematopoiesis, we investigated the ability of transduced vascular smooth muscle cells to provide therapeutic levels of G-CSF in rats.

MATERIALS AND METHODS

Retroviral vectors

The G-CSF expression vector was made by digesting rat G-CSF cDNA (Han, et al. 1996)with EcoRI and DraI and ligating the isolated DNA fragment (700bp) into the viral plasmid LXSN(Miller and Rosman 1989), previously digested with EcoRI and HpaI to provide the expression vector LrGSN. In LrGSN, rat G-CSF cDNA expression is driven by the strong viral LTR promoter and the neo resistance gene is expressed from SV-40 early region promoter/enhancer(Hock et al., 1989). From PA 317 packaging cell lines (Miller and Buttimore 1986) LrGSN had a viral titer of $8x10^6$ cfu/ml. The retroviral vector LASN, which encodes non-secreted human adenosine deaminase(ADA) (Hock, et al. 1989), was chosen as a control vector.

Cell Culture and transduction

Ecotropic PE501 and amphotropic PA317 retrovirus packaging cell lines (Miller, et al. 1986 ; Miller, et al. 1989) , NIH 3T3 thymidine kinase negative cells (Miller, et al. 1986), and primary cultures of rat vascular smooth muscle cells were grown in Dulbecco/Vogt-modified Eagle's medium (DMEM) with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum in humidified 5% CO_2/95% air at 37°C.

Rat smooth muscle cell cultures were prepared by enzymatic digestion of the aorta from male Fisher 344 rats. These cells were characterized by positive staining for muscle cell-specific actins with HHF35 antibody (Geary et al., 1994) while staining negative for von Willebrand factor (Geary, et al. 1994), an endothelial cell-specific marker. Early passage smooth muscle cells were exposed to 16 hr virus harvests from PA317-LrGSN and PA317-LASN amphotropic virus-producing cell lines for a period of 24 hr in the presence of polybrene (4ug/ml), and selected in G-418 antibiotic (1mg/ml).

G-CSF bioassay

Cytokine secretion from LrGSN-transduced cells was monitored using a murine cell line, NFS-60, that proliferates in response to G-CSF (Dale et al., 1992). Recombinant canine G-CSF (kindly supplied by Amgen, Thousand Oaks, CA) was used to construct a proliferation-response curve with murine NFS-60 cells (Dale, et al. 1992). In brief, short term proliferation was determined by measuring tritiated thymidine incorporation by cells seeded at a concentration of 10^5 cells/well in 96-well microtiter plates. Proliferative response to conditioned medium was measured after 24 hr at 37° C, 5% CO_2 and harvesting the cells 4 hr later on fiberglass filters using an automated cell harvester (Cambridge Technology, Cambridge, MA).

Cell Implantation

Male Fisher 344 rats (275-325 g) were premedicated with 0.04 mg/kg atropine sub-cutaneously. and 2.5 mg/kg enrofloxacin I.M. and were anesthetized using 44 mg/kg ketamine, 5 mg/kg xylazine, and 0.5 mg/kg acepromazine I.P. Animals were placed in dorsal recumbency and an incision was made along the ventral midline of the neck from the angle of the mandible to the thoracic inlet (Clowes, et al. 1994). The left common carotid and its internal and external branches were exposed using blunt dissection. Temporary ligatures were placed around the caudal common carotid artery and the cranial extent of the internal carotid. The external carotid was permanently ligated and an arterotomy made between this ligature and the bifurcation of the external and internal branches. A Fogarty 2F arterial embolectomy catheter was inserted through the arteriotomy site and the interior of the common carotid was balloon injured. The balloon catheter was withdrawn and a 24 gauge angiocatheter (Becton Dickinson) was inserted. $2x10^6$ transduced vascular smooth muscle cells were introduced into the lumen of the common carotid artery and allowed to seed the artery wall for 15 min. The angiocatheter was withdrawn and the arteriotomy site was closed by placing a second permanent

ligature around the external carotid artery just caudal to the site. Blood flow was re-established through the common carotid artery and the internal carotid artery and the incision was closed.

Blood Counts

Anticoagulated blood samples (100ul) were obtained from the tail vein with the animals under light ether anesthesia. Samples were obtained from 19 days before surgery and from 3 to 7 days post surgery for up to 7 weeks. Total WBC, platelets and hematocrit values were determined using a Coulter T-540 counter and differential WBC counts were obtained manually.

RESULTS

We monitored NFS-60 cell proliferation in the presence of conditioned medium from PA 317-LrGSN amphotropic packaging cells and LrGSN transduced Fisher rat vascular smooth muscle cells, using PA 317-LASN packaging cells and LASN transduced smooth muscle cells respectively to provide control medium. G-CSF expression was 2 ng/24 hr/10^7 cells from packaging cells and 8 ng/24 hr/10^7 cells from transduced smooth muscle cells. These assays indicate expression of a bioactive gene product from our retroviral vector, but are probably an underestimate because purified rat G-CSF was not available and recombinant canine G-CSF was used to generate a standard curve.

The absolute neutrophil counts of animals receiving LrGSN-transduced cells increased rapidly after cell implantation and by day 10 a relatively constant elevated plateau had been achieved which was sustained for at least 7 weeks(Figure 1). In contrast, neutrophil counts obtained before and after surgery from animals seeded with LASN-transduced cells did not show changes (Figure 1). This suggests that the consequences of surgery were not responsible for the increased number of neutrophils observed in the test rats. The surgical procedure was well tolerated by the animals, with no evidence of fever or other malaise and this may be reflected in the lack of neutrophil increases in animals receiving LASN-transduced cells. Pooled hematopoietic cell data from 6 control and 8 test rats are shown in Table 1. Neutrophil counts recorded from rats after LrGSN-cell implantation were significantly elevated over control rats receiving LASN-transduced cells ($P<0.001$). A similar statistical comparison of platelets, lymphocytes and red cells, as measured by hematocrit, showed no significant differences ($P>0.05$). This is noteworthy as previous studies of G-CSF administration to mice have shown reduction in red cell numbers(Molineux et al., 1990 ; Pojda et al., 1990).

DISCUSSION

We have shown that retrovirally-transduced vascular smooth muscle cells allow sustained expression of G-CSF that stimulated significantly elevated neutrophil production for periods of up to 7 weeks. In treated rats we documented mean increases of 1,800 neutrophils/ul, which would be a therapeutic cell number for patients with severe chronic neutropenia or cyclic hematopoiesis. In these patients and cylic neutropenic dogs provision of neutrophil counts in excess of 500/ul prevent severe recurrent infection and would be therapeutic(Lothrop, et al. 1988 ; Hammond, et al. 1989 ; Morstyn, et al. 1993).

We observed no significant differences in lymphocyte and platelet numbers and hematocrit between animals treated with G-CSF expressing cells and controls, indicating vector-encoded G-CSF production stimulated neutrophil production without other hematological effects. This is of interest because previous studies in mice have shown that G-CSF administration caused reduced erythropoiesis and anemia (Molineux, et al. 1990 ; Pojda, et al. 1990). As long-term administration of recombinant G-CSF to humans (Morstyn et al., 1988 ; Morstyn, et al. 1993), and dogs(Hammond, et al. 1989 ; Hammond, et al. 1990) specifically stimulates neutrophil production, our data suggest that rats provide a more appropriate human model for the physiological study of G-CSF administration than mice. G-CSF expression, unlike GM-CSF (Johnson, et al. 1989) and IL-3 (Wong, et al. 1989), did not cause pathological changes in hematopoiesis. In previous studies these latter cytokines produced a fatal myeloproliferative syndrome in mice (Wong, et al. 1989) (Johnson, et al. 1989) .

The implantation of genetically modified vascular smooth muscle cells to patients will require an approach other than arterial seeding. We recently proposed the seeding of synthetic PTFE (polytetrafluoroethylene) grafts as a method to return transduced vascular smooth muscle cells to their donor (Geary, et al. 1994) and this technique can be readily studied in collie dogs with cyclic hematopoiesis, an appropriate clinical model for

affected patients (Dale, et al. 1988 ; Lothrop, et al. 1988). We have shown that the rat carotid artery seeding procedure resulted in approximately 10^5 cells being retained within the artery(Osborne, et al. 1995). Based on this finding we estimate that the potentially therapeutic level of neutrophils we observed in rats was derived from 10^5 transduced cells . We previously estimated that 10^8 transduced cells can be seeded into a 10 cm x 4 mm PTFE graft (Osborne et al., 1993 ; Geary, et al. 1994) and from the current study, this should provide a therapeutic level of G-CSF to a dog. It is also probable that neutropenic dogs and patients are more sensitive to G-CSF than normals.

Our data show that transduced vascular smooth muscle cells do not inactivate retroviral vector sequences, in agreement with previous studies of retrovirally-mediated gene expression in these target cells(Lynch, et al. 1992 ; Clowes, et al. 1994 ; Osborne, et al. 1995). This is in contrast to skin fibroblasts where vector inactivation has been documented in both rats(Palmer et al., 1991) and dogs(Ramesh et al., 1993). Thus, data is accumulating to show that vascular smooth muscle cells provide an ideal target tissue for gene therapy. These cells are readily obtained, cultured, transduced and returned to their donor. Implantation of these cells in the blood circulation suggests their use for the secretion of not only hormones but also clotting factors for the treatment of patients with hemophilia and enzymes for treatment of lysosomal storage disorders.

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FIGURE LEGEND

FIG. 1. Effect of seeding transduced vascular smooth muscle cells on absolute neutrophil count (ANC). Open symbols, animals implanted with LrGSN-transduced cells; solid symbols, animals receiving LASN-transduced cells. Surgery was on day zero.

Table 1. Control and treated rat blood counts

		Neutrophils per µl	Lymphocytes per µl	HCT	Platelets per µl ($\times 10^{-3}$)
LASN	Pre-surgery	1850 ±390	6870 ±1180	43.7 ±2.84	673 ±167
(n=6)	Post-surgery	1870 ±460[a]	6180 ±970[b]	45.0 ±2.22[b]	716 ±96[b]
LrGSN	Pre-surgery	1880 ±750	6470 ±1140	44.3 ±2.38	678 ±122
(n=8)	Post-surgery	3670 ±740[a]	6240 ±1300[b]	43.9 ±1.55[b]	704 ±94[b]

[a] $P<0.001$

[b] $P>0.05$

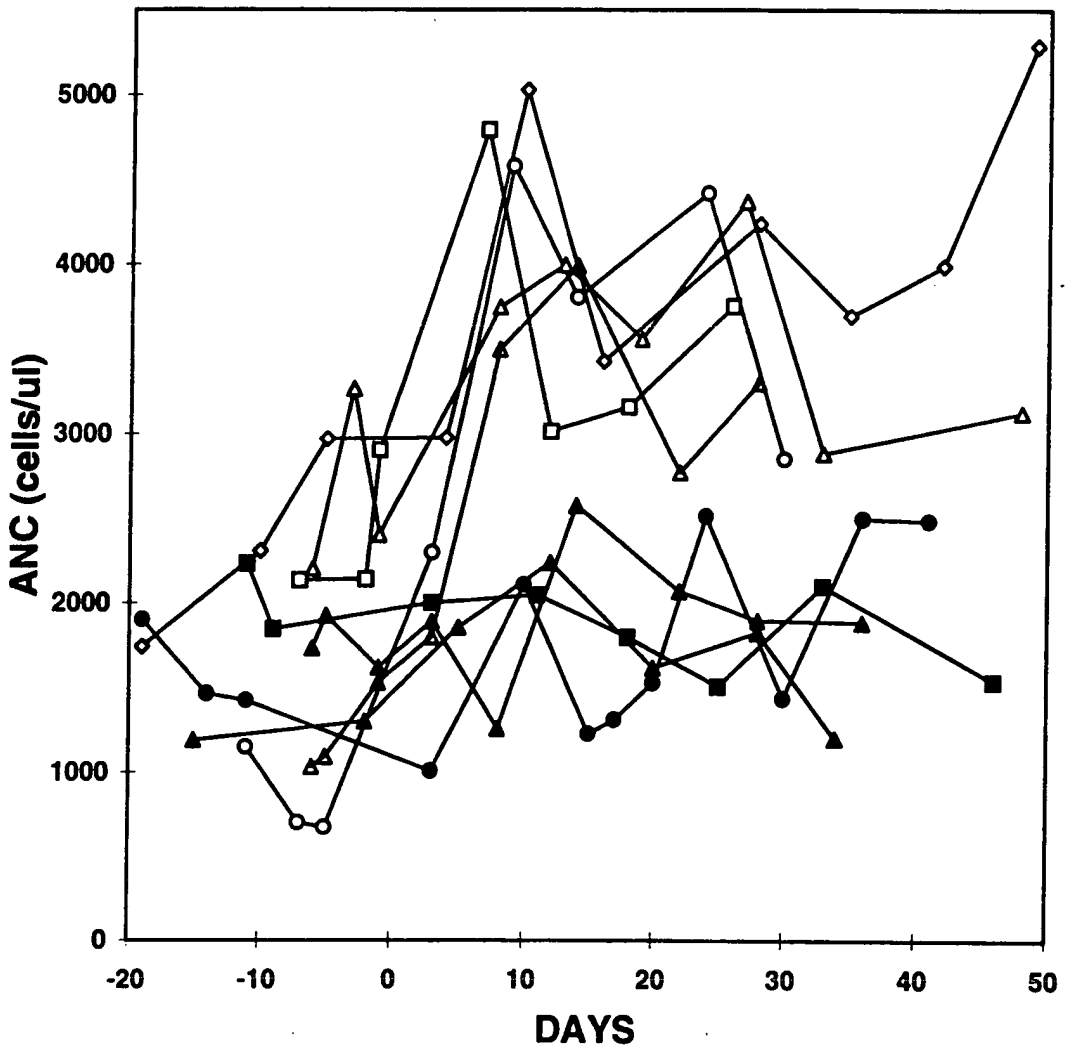
ANC (cells/ul)
5000
4000
3000
2000
1000
0
-20
-10
0
10
20
30
40
50
DAYS

Table 1. Control and treated rat blood counts

		Neutrophils per µl	Lymphocytes per µl	HCT	Platelets per µl ($x 10^{-3}$)
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[a] P<0.001

[b] P>0.05